

Monitoring of Fecal Pollution in Coastal Waters by Use of Rapid Enzymatic Techniques

LIV FIKSDAL,^{1*} MONIQUE POMMEPUY,² MARIE-PAULE CAPRAIS,² AND INGUNN MIDTTUN¹

Department of Hydraulic and Environmental Engineering, The Norwegian Institute of Technology, University of Trondheim, 7034 Trondheim, Norway,¹ and Laboratory of Microbiology, Institut Français de Recherche pour l'Exploitation de la Mer, Centre de Brest, 29280 Plouzane, France²

Received 5 November 1993/Accepted 10 March 1994

Enzyme assays for 4-methylumbelliferyl- β -D-galactopyranosidase and 4-methylumbelliferyl- β -D-glucuronidase activities were used for rapid detection (25 min) of fecal water pollution and to determine the impact of sewage discharge in coastal waters. Two coastal areas were investigated: (i) an estuary characterized by a high degree of contamination downstream of a discharge from a sewage treatment plant and a low degree of water renewal and (ii) a fjord with a low degree of pollution and a high degree of water renewal. Statistical analysis showed that a global correlation curve could be used to estimate concentrations of culturable fecal coliform bacteria in the two coastal areas, although environmental factors important for cell physiology (e.g., salinity) varied at different sampling locations. The sensitivity limit for detection of 4-methylumbelliferyl- β -D-glucuronidase activity corresponded to bacterial concentrations on the order of 10 to 100 CFU/100 ml. The 4-methylumbelliferyl- β -D-galactopyranosidase assay was less sensitive because of a higher rate of substrate autohydrolysis. The detection limit corresponded to bacterial concentrations on the order of 100 to 1,000 fecal coliforms per 100 ml.

Coastal waters are often used for multiple purposes, i.e., as recipients for wastewaters, for recreation, and for aquaculture. Discharges of sewage effluents have a negative impact on the coastal environment and make it less attractive for recreation as well as for aquaculture, which is sensitive to the presence of pathogenic microorganisms and toxic compounds. Occasional pollution by effluents containing human pathogens may result in prohibition of the sale of shellfish and economic loss.

In these areas, water quality has to be monitored. Standard methods for assessing the hygienic quality of water, using either membrane filtration or most-probable-number techniques, require 24 to 72 h to complete. The elapsed time is too great to warn of substandard quality that may require immediate remedial measures.

Fluorogenic methylumbelliferyl (MU) substrates have been used for the last 20 years in bacterial diagnostics for detection of coliform bacteria by enzyme assays (2, 12, 16). 4-MU- β -D-galactopyranoside (MUGal) and 4-MU- β -D-glucuronide (MUGlu) are hydrolyzed by the enzymes β -D-galactosidase and β -D-glucuronidase, present in coliform and fecal coliform (FC) bacteria, respectively. MU substrates have been used to detect sewage contamination in fresh water (3, 6, 7, 20) as well as in marine waters (13, 21).

Many so-called rapid enzymatic tests require high densities of bacteria, and the completion times are, in practice, around 24 h, almost the same amount of time required for conventional tests. An objective of the present work was to investigate bacterial water quality in coastal areas by use of rapid enzymatic hydrolysis of MUGal and MUGlu. The enzyme assay (25 min) has previously been applied to freshwater samples with MUGal as substrate (3), while MU-heptanoate has been used to enumerate pure cultures of *Escherichia coli* in seawater (11).

A second objective was to evaluate the use of this method in marine waters with MUGal and MUGlu as substrates.

MATERIALS AND METHODS

Sampling locations. The water quality of the Morlaix River and Estuary, located in Bretagne, France, was investigated on 27 April 1992 (neap flood tide) and 4 May 1992 (spring flood tide). Stations -2, -1, and 0 represent river upstream of a biological treatment plant, effluent after primary treatment, and effluent after biological treatment, respectively. The biological oxygen demand, chemical oxygen demand, and suspended solids loading rates were 1,030 kg of O per day, 3,100 kg of O per day, and 4,590 kg/day, respectively, while discharges were 70 kg of O per day, 470 kg of O per day, and 145 kg/day. Salinities at different sites in the Morlaix River and Estuary increased from 3‰ (station -2) to 40‰ (station 9). Water quality of the Bjugn Bay and Estuary, near Trondheim, Norway, was investigated on 25 August 1992 (flood and ebbing tides) and 31 August 1992 (ebb and flooding tides). The bay receives primary treated sewage. The biological oxygen demand, chemical oxygen demand, and suspended solids loading rates were 85 kg of O per day, 170 kg of O per day, and 120 kg/day, respectively, while discharges were 70 kg of O per day, 135 kg of O per day, and 100 kg/day, respectively. Station 3.3 was in the bay, above the sewage outlet; station 3.6 was located in the treatment plant (effluent after primary treatment). Salinities at different sites in the Bjugn Bay varied from 27‰ to 31‰ (stations 3, 3.3, 4, 5).

FC bacteria. FC bacteria were recovered by membrane filtration (Millipore filter GA, 47-mm diameter, 0.22- μ m pore diameter) on MacConkey agar medium (Difco), and CFU were counted after 24 h of incubation at 44.5°C.

β -D-Galactosidase (MUGalase) assay. Water samples were filtered through a 0.22- μ m-pore-size, 47-mm-diameter membrane filter (Millipore GTTP). The filter was then aseptically placed in a 250-ml flask containing 20 ml of sterile 0.05 M phosphate buffer (pH 7.9) and 0.05% sodium lauryl sulfate

* Corresponding author. Mailing address: Department of Hydraulic and Environmental Engineering, NTH, University of Trondheim, 7034 Trondheim, Norway. Fax: 47-7-591298. Electronic mail address: Liv.Fiksdal@IVB.UNIT.NO.

(Sigma Chemical Co.). MUGal (Sigma Chemical Co.) was added at a final concentration of 0.4 mg/ml to each flask containing 20 ml of buffer plus sodium lauryl sulfate and to a sterile control flask containing 20 ml of buffer plus 0.05% sodium lauryl sulfate. The flasks were incubated in a shaking water bath at 44.5°C, and the fluorescence intensity of sample aliquots was measured every 5 min for 25 min with a Perkin-Elmer 3000 spectrofluorometer (excitation at 365 nm, emission at 440 nm) after addition of 100 μ l of 0.1 M NaOH to a 2.5-ml sample in the cuvette to obtain pH >10 and maximum MU fluorescence (5). At least two hydrolysis control flasks (blanks) were included per batch of 10 samples. The enzymatic activity measured as the production rate of MU (micromolar concentration per minute) was determined by least-squares linear regression.

β -D-Glucuronidase (MUGluase) assay. The MUGluase assay was done as described for the MUGal assay, except filters were placed in flasks containing 17 ml of sterile 0.05 M phosphate buffer (pH 6.4) and the assay was started by addition of 3 ml of MUGlu solution (50 mg of MUGlu–50 ml of sterile Triton water). Triton water was prepared by adding 1 drop of Triton X-100 to 50 ml of distilled water. At least two hydrolysis control flasks (blanks) were included per batch of 10 samples.

Optimization of MUGluase assay. Optimization of the MUGlu concentration was carried out by using a pure culture of *E. coli* H10407 (from isolates of Evans et al. [10] provided by B. Joly, University of Clermont-Ferrand, Clermont-Ferrand, France). The bacteria were grown in tryptone-soya broth (Oxoid), harvested by centrifugation, washed two times in saline, and resuspended in autoclaved (120°C, 20 min) seawater. The enzyme assay was then performed as described for the MUGluase assay, at various pHs, concentrations of MUGlu, temperatures, and fluorescence emission wavelengths.

RESULTS

Optimization of enzyme assay. Results indicated that enzymatic activities for MUGal substrate corresponded to saturation kinetics, although an apparent V_{\max} was not reached. A modest increase in the rate of the MUGluase-catalyzed reaction as a function of substrate concentration was demonstrated; the logarithm of velocity of substrate hydrolysis increased from $-2.16 \log \mu\text{M MU per min}$ to $-2.00 \log \mu\text{M MU per min}$ when the concentration of MUGlu increased from 0.07 to 0.20 g/liter. The rate of hydrolysis at 0.15 g of MUGlu per liter was only 4% lower than the maximum value, and 0.15 g of MUGlu per liter was used during the coastal water investigations. It is recognized that the kinetics of the pure *E. coli* experiment may not be representative for the stressed FCs found in seawater. Nonetheless, the concentration of MUGlu used in the field experiments was deemed to be acceptable for the purposes of detection of the FCs. The hydrolysis rate (micromolar concentration of MU per minute) decreased by 30% as pH increased from 6.4 to 7.2. A similar reduction was observed when the assay temperature was decreased from 44.5°C to 41.5°C. On the basis of these results, a pH of 6.4 and an incubation temperature of 44.5°C were used during the MUGlu assay. The rate of fluorescence increased 13% (at pH 6.4 and 44.5°C) when the emission wavelength was decreased from 465 nm to 440 nm.

To avoid substrate limitation in heavily contaminated samples (sewage effluents), the concentration of MUGal was increased from the 0.05 mg/ml used previously (3) to 0.4 g/ml.

Rapid detection of sewage pollution of coastal water. Salinities at sampling locations in the Morlaix River and Estuary

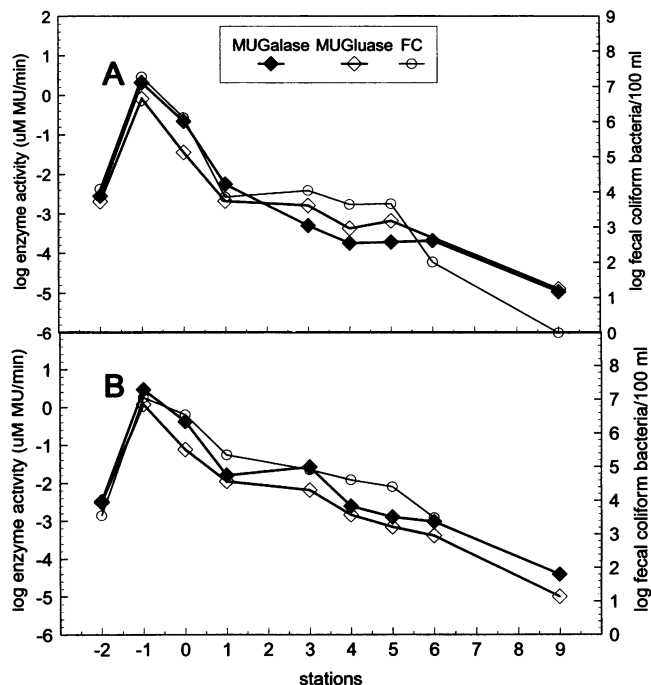


FIG. 1. Enzyme activity and number of FC bacteria in the Morlaix River and Estuary on 27 April 1992 (A) and 4 May 1992 (B). Salinities at the different sites increased from 3‰ (station -2) to 40‰ (station 9).

varied, from brackish to oceanic water. The Bjugn Bay and Estuary are open to the North Sea of the Atlantic Ocean, and salinities were generally high.

Numbers of FCs and enzyme activities of MUGalase and MUGluase in the Morlaix River decreased with increased distances from the water treatment plant (Fig. 1). In the Bjugn Bay and Estuary, small local outlets of sewage in addition to the treatment plant discharge resulted in a less clear relationship between the degree of water pollution and the distance from the sewage treatment plant than in the Morlaix area (Fig. 2). Enzyme activity was generally not detected when numbers of FCs were less than 10 to 100 CFU/100 ml. On one occasion (at station 9, Morlaix), however, hydrolysis of MUGal and MUGlu was registered at unexpectedly low levels of culturable FC bacteria (<1 and 1 CFU/100 ml).

The separate data sets from Morlaix and Bjugn for MUGalase activity versus FCs were each fit to a line by using weighted least-squares analysis. The slope and intercept, respectively, of each fit were tested with an *F* test, and they were found to be the same (fits were done with and without the results from station 9, Morlaix, because of inherent uncertainties with low plate counts, e.g., 1 FC/100 ml). The significance level of the test was set at $P < 0.05$. Because of similar potential bacterial population of discharges and similar potential bacterial behavior in receiving waters, the two data sets, including that from station 9, Morlaix, were then combined (Fig. 3), and least-squares analysis was used to obtain the global slope and intercept. The same statistical procedure was applied to data sets for MUGluase versus FCs. The slope and intercept of the Morlaix and Bjugn fits were found to be the same when data from station 9, Morlaix, were included, and the data were therefore combined (Fig. 3). However, without data from station 9, the two data sets appeared to obey different regression parameters (not shown).

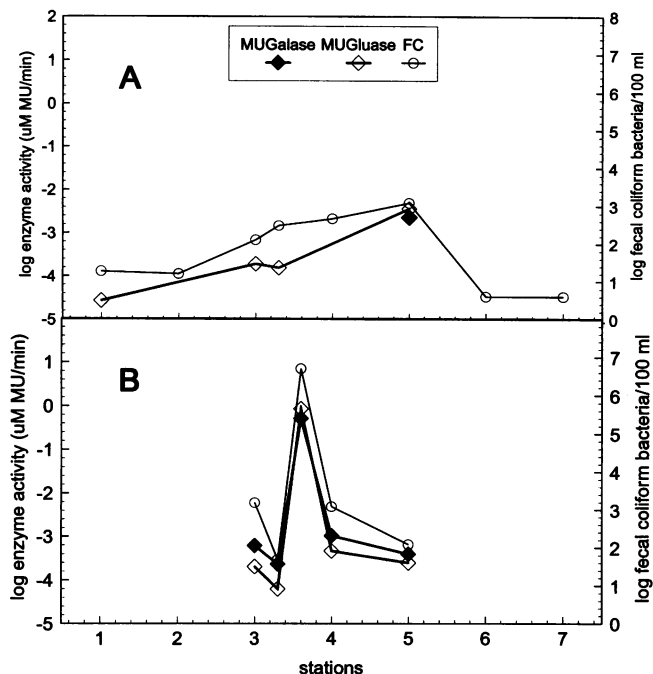


FIG. 2. Enzyme activity and number of FC bacteria in the Bjugn Bay and Estuary on 25 August 1992 (A) and 31 August 1992 (B) (sewage effluent, station 3.6, was not analyzed for panel A). Salinities at the different sites in the bay (B) varied from 27‰ to 31‰ (station 3.6 was not analyzed).

The logarithmic presentation of numbers of FCs versus MUGalase and MUGluase activities, respectively, for all locations of Morlaix and Bjugn (Fig. 3) demonstrated good linear correlations (correlation coefficients of $r = 0.87$ and $r = 0.88$ for MUGalase and MUGluase, respectively).

Enzyme activity will be a linear function of cell number (i.e., the enzyme activity per cell is constant) only when the slope of the linear logarithmic data curve is equal to 1. This was not the case in the present work, and it can be demonstrated by using regression line coefficients (Fig. 3) to calculate enzymatic activities per culturable cell that this activity seemed to decrease as the numbers of cells increased.

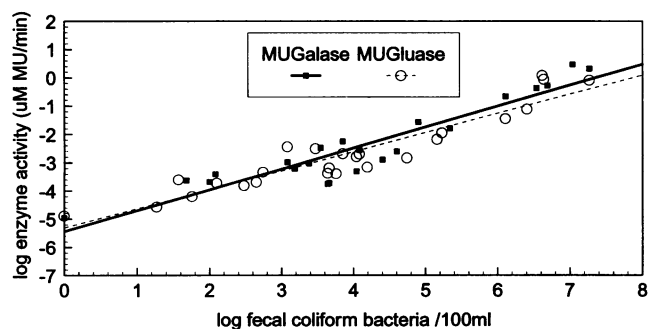


FIG. 3. MUGalase and MUGluase activity versus number of FC bacteria (all data from Bjugn and Morlaix sites). Regression lines: $\log y_{\text{MUGalase}} = 0.74 \log x - 5.44$; $\log y_{\text{MUGluase}} = 0.68 \log x - 5.31$.

DISCUSSION

The bacteriological quality of drinking water has recently been investigated by several authors using kits for simultaneous detection of total and FC bacteria. The kits rely on enzymatic conversion of *o*-nitrophenyl- β -galactopyranoside by coliform bacteria and MUGlu by FC bacteria in 24 h (6, 7, 9, 20). Some studies have indicated that injured FCs in drinking water may be recovered to a lower degree with this technique than with standard methods (6). Recently, other authors have found such kits to be useful for detection of *E. coli* exposed to chlorine in drinking water (8, 19) and for *E. coli* exposed to environmental stress in coastal water (21).

We have investigated the use of MUGal and MUGlu in separate, rapid assays for detection of fecal pollution of coastal water. Because of their short assay time (25 min), the enzymatic methods described in the present work are candidate methods for early warning of sewage pollution of water. The investigations were carried out to study the impact of sewage discharge on bacteriological water quality and the use of rapid techniques for assessing bacteriological contamination of seawater.

Two coastal areas were selected for the study; an estuary (Morlaix) where mixing of sewage discharge and contaminated fresh water with seawater is complex, as demonstrated previously by use of mathematical models (26), and a fjord (Bjugn) where the sewage discharge is rapidly mixed with seawater. In Morlaix Estuary, there was a clear impact of the sewage discharge 4 km downstream of the outlet (until sampling station 5) at high tide as well as at low tide. In this part of the estuary, bacterial numbers and enzymatic activities were both high, and the enterobacterial die-off rate has previously been shown to be low (time for 90% of bacteria to lose culturability, 9 to 10 days) because of high concentrations of soluble and particulate organic matter which increase bacterial salt tolerance and prevent light penetration and the solar bactericidal effect (22, 26). In the outer part of the estuary (station 9), the time for 90% of bacteria to lose culturability is shorter (2 h to 2 days), and physical dilution processes efficiently reduce bacterial numbers. Dilution processes have been demonstrated to be more important than environmental factors affecting cell physiology, for numbers of culturable bacteria in the Morlaix Estuary (26). In the Bjugn Fjord, the combination of a small amount of sewage discharge (1,200 person equivalents) and a high degree of mixing with seawater resulted in limited impact by the discharge. The water was polluted near the sewage outlet; at distances >500 m from the outlet, high salinities were associated with low levels of FCs and enzymatic activity.

In the present work, MUGluase activity corresponding to bacterial concentrations in the order of 10 to 100 FCs/100 ml could generally be detected. The higher rate of substrate autohydrolysis in the MUGalase assay affected the detection limit, which corresponded to bacterial concentrations on the order of 100 to 1,000 FCs/100 ml.

In addition to the limit on the precision of the rapid assays mentioned above, other sources of error are also possible. Galactosidase-negative coliforms and galactosidase-positive noncoliforms, as well as glucuronidase-negative FCs and glucuronidase-positive non-FCs, may affect the enzyme assays (6, 7, 12, 16, 25). The lack of a relationship between the intensity of autoradiographic signals of the *uidA* gene encoding β -glucuronidase enzyme and the expression of enzyme activity in MUGlu media has recently been demonstrated for *E. coli* isolated from treated and raw water sources (17). Martins et al. (17) suggested that the physiological condition of the bacteria could have been a variable responsible for the nonexpression

of the enzyme activity of the inducible β -glucuronidase enzyme.

Because enzyme activities are subject to the physiological status of the bacteria, a variable fraction of the coliform bacteria may be stressed when changes in irradiation, salinity, temperature, and nutrient concentration of the environment occur (22). Interestingly, when results from the present study are used to calculate enzymatic activity per culturable bacterium, enzyme activity seems to increase when the concentration of cells in the receiving waters decreases. The increase in enzyme activity could have been a response to environmental changes; starvation studies of bacteria have, for instance, indicated an increase in the concentration of substrate-capturing enzymes as well as induction of high-affinity uptake systems for carbon substrates during starvation (15, 18). In our study, the apparent increase in MUGalase and MUGlucase activity per culturable cell could, however, also have occurred as a result of an increase of the fraction of viable, nonculturable bacteria. Viable, nonculturable bacteria have been shown to be metabolically active, even if they cannot be detected by commonly used cultivation techniques (1, 23, 24). Several studies have demonstrated a decrease in the relationship between culturable and viable enterobacteria during exposure to environmental stress (1, 4, 14).

Although environmental factors (e.g., salinity, irradiation, nutrient concentration) varied at different sampling locations in the present investigation and enzyme activity per bacterial cell may have varied along with a variable fraction of culturable to viable cells, the good linear correlation between logarithms of enzyme activities and concentrations of culturable FC bacteria encourages continued use of the rapid enzyme assays for monitoring of sewage pollution in marine waters. Further investigations of how marine environments affect the MUGalase or MUGlucase activity of FC bacteria would be important for a more extensive evaluation of the use of present methods for detection of culturable and viable, nonculturable coliform bacteria.

ACKNOWLEDGMENTS

We thank N. Haget for assistance with assay-optimizing experiments and J. S. Tyssedal for help with statistical analysis.

This work was partially supported by a grant to L.F. from IF-REMER. Financial support was also received from the Cultural Agreement between Norway and France as a travel grant to M. Pommeupuy.

REFERENCES

1. Barcina, I., J. M. González, J. Irriberri, and L. Egea. 1989. Effect of visible light on progressive dormancy of *Escherichia coli* cells during the survival process in natural fresh water. *Appl. Environ. Microbiol.* **55**:246–251.
2. Bascomb, S. 1987. Enzyme tests in bacterial identification. *Methods Microbiol.* **19**:105–160.
3. Berg, J. D., and L. Fiksdal. 1988. Rapid detection of total and fecal coliforms in water by enzymatic hydrolysis of 4-methylumbelliferyl- β -D-galactoside. *Appl. Environ. Microbiol.* **54**:2118–2122.
4. Byrd, J. J., H.-S. Xu, and R. R. Colwell. 1991. Viable but nonculturable bacteria in drinking water. *Appl. Environ. Microbiol.* **57**:875–878.
5. Chrost, R. J., and H. J. Krambeck. 1986. Fluorescence correction for measurement of enzyme activity in natural waters using methylumbelliferyl substrates. *Arch. Hydrobiol.* **106**:79–90.
6. Clark, D. L., B. B. Milner, M. H. Stewart, R. L. Wolfe, and B. H. Olson. 1991. Comparative study of commercial 4-methylumbelliferyl- β -D-glucuronide preparations with the *Standard Methods* membrane filtration fecal coliform test for the detection of *Escherichia coli* in water samples. *Appl. Environ. Microbiol.* **57**:1528–1534.
7. Clark, J. A., and H. El-Shaarawi. 1993. Evaluation of commercial presence-absence test kits for detection of total coliforms, *Escherichia coli*, and other indicator organisms. *Appl. Environ. Microbiol.* **59**:380–388.
8. Covert, T. C., E. W. Rice, S. A. Johnson, D. Berman, C. H. Johnson, and P. J. Mason. 1992. Comparing defined substrate coliform tests for the detection of *Escherichia coli* in water. *J. Am. Water Works Assoc.* **84**:98–104.
9. Edberg, S. C., M. J. Allen, D. B. Smith, and the National Collaborative Study. 1988. National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: comparison with the standard multiple tube fermentation method. *Appl. Environ. Microbiol.* **54**:1595–1601.
10. Evans, D. G., D. J. Evans, Jr., and W. Tjoa. 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. *Infect. Immun.* **18**:330–337.
11. Fiksdal, L., M. Pommeupuy, A. Derrien, and M. Cormier. 1989. Production of 4-methylumbelliferyl heptanoate hydrolase by *Escherichia coli* exposed to seawater. *Appl. Environ. Microbiol.* **55**:2424–2427.
12. Frampton, E. W., and L. Restaino. 1993. Methods for *Escherichia coli* identification in food, water and clinical samples based on beta-glucuronidase detection. *J. Appl. Bacteriol.* **74**:223–233.
13. Gauthier, M. J., M. C. Toregossa, M. C. Babelona, R. Cornax, and J. J. Borrego. 1991. An intercalibration study of the use of 4-methylumbelliferyl- β -D-glucuronide for the specific enumeration of *Escherichia coli* in sea water and marine sediments. *Syst. Appl. Microbiol.* **14**:183–189.
14. Grimes, D. J., and R. R. Colwell. 1986. Viability and virulence of *Escherichia coli* suspended by membrane chamber in semitropical ocean water. *FEMS Microbiol. Lett.* **34**:161–165.
15. Kjelleberg, S., M. Hermansson, P. Marden, and G. Jones. 1987. The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. *Annu. Rev. Microbiol.* **41**:25–49.
16. Manafi, M., W. Kneifel, and S. Bascomb. 1991. Fluorogenic and chromogenic substrates used in bacterial diagnostics. *Microbiol. Rev.* **55**:335–348.
17. Martins, M. T., I. G. Rivera, D. L. Clark, M. H. Stewart, R. L. Wolfe, and B. H. Olson. 1993. Distribution of *uidA* gene sequences in *Escherichia coli* isolates in water sources and comparison with the expression of β -glucuronidase activity in 4-methylumbelliferyl- β -D-glucuronide media. *Appl. Environ. Microbiol.* **59**:2271–2276.
18. Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz. 1989. Genetic basis of starvation survival in nondifferentiating bacteria. *Annu. Rev. Microbiol.* **43**:293–316.
19. McCarty, S. C., J. H. Standridge, and M. C. Stasiak. 1992. Evaluating a commercially available defined-substrate test for recovery of *E. coli*. *J. Am. Water Works Assoc.* **84**:91–95.
20. Olson, B. H., D. L. Clark, B. B. Milner, M. H. Stewart, and R. L. Wolfe. 1991. Total coliform detection in drinking water: comparison of membrane filtration with Colilert and Coliquik. *Appl. Environ. Microbiol.* **57**:1535–1539.
21. Palmer, C. J., Y.-L. Tsai, A. Lee Lang, and L. R. Sangermano. 1993. Evaluation of Colilert-Marine Water for detection of total coliforms and *Escherichia coli* in the marine environment. *Appl. Environ. Microbiol.* **59**:786–790.
22. Pommeupuy, M., J. F. Guillard, E. Duprey, A. Derrien, F. Le Guyader, and M. Cormier. 1992. Enteric bacteria survival factors. *Water Sci. Technol.* **25**:93–103.
23. Roszak, D. B., and R. R. Colwell. 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* **51**:365–379.
24. Roszak, D. B., and R. R. Colwell. 1987. Metabolic activity of bacterial cells enumerated by direct viable count. *Appl. Environ. Microbiol.* **53**:2889–2893.
25. Rychert, R. C., and G. R. Stephenson. 1986. Lactose negative *Escherichia coli* from rangeland streams: source, antibiotic resistance, and colicinogenicity. *Water Res. Bull.* **22**:39–42.
26. Salomon, J. C., and M. Pommeupuy. 1990. Mathematical model of bacterial contamination of the Morlaix estuary (France). *Water Res.* **24**:983–994.